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CHAPTER 6

ELUCIDATING THE ROLE OF PROTEIN SYNTHESIS IN HIPPOCAMPUS-DE- PENDENT MEMORY CONSOLIDATION ACROSS THE DAY AND NIGHT

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Abstract

It is widely acknowledged that de novo protein synthesis is crucial for the formation and consolidation of long-term memories. While the basal activity of many signaling cascades that modulate protein synthesis fluctuate in a circadian fashion, it is unclear whether the temporal dynamics of protein synthesis-dependent memory consolidation varies depending on the time of day. More specifically, it is unclear whether protein synthesis inhibition affects hippocampus-dependent memory consolidation in rodents differentially across the day (i.e., the inactive phase with an abundance of sleep) and night (i.e., the active phase with little sleep). To address this question, male and female C57Bl6/J mice were trained in a contextual fear conditioning task at the beginning or the end of the light phase. Animals received a single systemic injection with the protein synthesis inhibitor anisomycin or vehicle directly, 4 hours, 8 hours, or 11.5 hours following training, and memory was assessed after 24 hours. Here, we show that protein synthesis inhibition impaired the consolidation of context-fear memories selectively when the protein synthesis inhibitor was administered at the first three time points, irrespective of timing of training. Even though the basal activity of signaling pathways regulating de novo protein synthesis may fluctuate across the 24h cycle, these results suggest that the temporal dynamics of protein synthesis-dependent memory consolidation are similar for day-time and night-time learning.

Keywords: Anisomycin, hippocampus, memory, mice, protein synthesis

Introduction

The capacity to form new memories is crucial for an animals' adaptation to a complex and often changing environment (Brüel-Jungferman et al., 2007). The formation of new memories involves a wide range of molecular and cellular processes (Nadel et al., 2012; Asok et al., 2019). Memory processes can be divided into different phases and include: (1) the acquisition of new information, (2) the consolidation of short-term into long-term memories, and (3) the retrieval of the stored information (Abel & Lattal, 2001). These processes occur in different time frames and depend on different molecular mechanisms, although some of the underlying mechanisms may overlap (Abel & Lattal, 2001; Akkerman et al., 2016). The initial acquisition of sensory input takes place at the moment when new information is presented and includes the encoding of this information (Abel & Lattal, 2001; Tonegawa et al., 2015). Memory consolidation starts directly following acquisition (Abel & Lattal, 2001; Havekes et al., 2015). During this process, the initial labile memory is stabilized into a long-term memory, and this process is sensitive to disruption (Abel & Lattal, 2001; Havekes et al., 2015). Subsequently, when necessary, the stored information can be accessed and recalled during retrieval (Abel & Lattal, 2001; Havekes et al., 2015).

The hippocampus critically contributes to the formation of declarative and episodic memories including spatial and contextual memories (Scoville & Milner, 1957; Morris et al., 1982; Phillips & LeDoux, 1992; Squire, 1992; Nadel & Moscovitch, 1997; Moser & Moser, 1998; Daumas et al., 2005; Oliveira et al., 2010; Eichenbaum & Cohen, 2014). A frequently used paradigm to elucidate the molecular underpinnings of context-specific memories is the contextual fear conditioning (CFC) task, which relies on both the hippocampus as well as the amygdala (Phillips & LeDoux, 1992; Rudy et al., 2002; Daumas et al., 2005; Parsons et al., 2006; Kochli et al., 2015; Havekes et al., 2016a). Training in this paradigm results in a context-fear association of the conditioning box (the conditioned stimulus, CS) with an unexpected adverse stimulus, often a mild foot shock (the unconditioned stimulus, US). Upon re-exposure to the same context, mice that successfully learned to associate the CS with the US show high levels of freezing (i.e., the complete lack of movement except for respiratory behavior), indicating a context-fear memory. As only a single training session is needed to form a robust contextual-fear memory, this test is ideally suited to examine the molecular mechanisms contributing to the

different memory stages.

The consolidation of stable, naturally retrievable long-term memories including those dependent on proper hippocampal function, require *de novo* protein synthesis (Davis & Squire, 1984; Bourtchouladze et al., 1998; Lattal et al., 2004; Jarome & Helmstetter, 2014; Ryan et al., 2015). Indeed, blocking protein synthesis using protein synthesis inhibitors attenuates the formation of object memories (Rossato et al., 2007), spatial memories (Artinian et al., 2008), and context-fear associations (Bourtchouladze et al., 1998). Interestingly, the regulation of processes involved in learning and memory, such as protein synthesis, may vary across the day and night (Ramm & Smith, 1990; Nakanishi et al., 1997; Gerstner & Yin, 2010; Jilg et al., 2010; Frank, 2016; Rawashdeh et al., 2016; Aten et al., 2018; Snider et al., 2018; Kim et al., 2019). For example, hippocampal cAMP levels as well as the phosphorylation of mitogen-activated protein kinase (MAPK), and cAMP response element-binding protein (CREB) show daily oscillations, which may suggest circadian regulation of cAMP/MAPK/CREB pathways (Mizuno & Giese, 2005; Trifilieff et al., 2006; Eckel-Mahan et al., 2008; Rawashdeh et al., 2016; Snider et al., 2018). Importantly, the regulation of mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, which is key for protein synthesis by initiating translation, also shows daily oscillations (Jouffe et al., 2013; Saraf et al., 2014; Robles et al., 2017). Recently, it was shown that accumulation of Per2, a core clock protein, results in the inactivation of mTORC1, decreasing translation of proteins (Wu et al., 2019). Hence, the regulation of processes that are important for learning and memory show oscillations across the day and night.

Despite these observations, it is unclear whether these daily oscillations in the basal activity of signaling pathways critical for learning and memory affect the temporal dynamics of protein synthesis-dependent memory consolidation. In other words, does daily variation in the basal activity of these pathways mean that the processing and storage of new information that depends on protein synthesis also varies across the day? To answer this question, we assessed in mice whether inhibition of protein synthesis affects memory consolidation similarly during the light phase (the resting phase) and dark phase (the active phase). Animals were trained at the beginning or end of the light phase, and the protein synthesis inhibitor anisomycin or vehicle was

systemically delivered at different time points following training in the hippocampus-dependent contextual fear conditioning task.

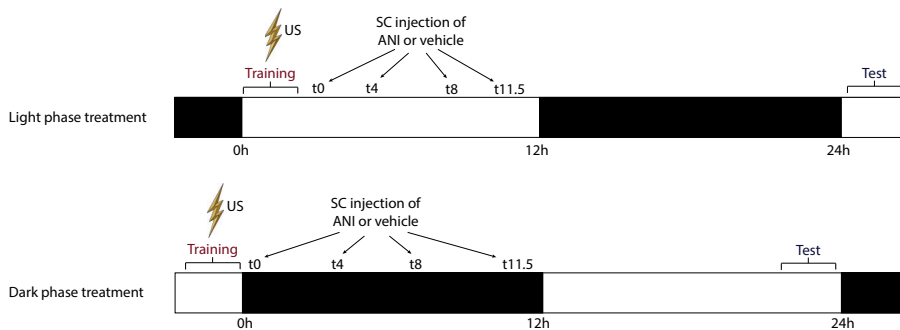
Materials and methods

Subjects

A total of 64 male and 64 female C57BL/6J mice were obtained at 6 weeks of age (Janvier Labs), and housed in same-sex pairs throughout the experiment. Cages contained a cardboard roll, standard bedding and nesting material. Food and water were available ad libitum. During the experiment, the mice were housed under constant temperature (21 °C) and on a 12 h light/12 h dark schedule with lights on at 10 am for animals that were trained at the beginning of the light phase, and lights on at 10 pm for animals trained at the end of the light phase (see next paragraph). Experiments were performed when the animals were 12-16 weeks old. All described procedures were approved by the national Central Authority for Scientific Procedures on Animals (CCD) and the Institutional Animal Welfare Body (IvD, University of Groningen, The Netherlands).

Experimental design

This study consisted of 16 groups of mice, each with a total of 7-8 animals (equal males and females) and animals were randomly assigned to the groups. Half of the groups was trained in the CFC task at the beginning of the light phase, i.e., at the beginning of their circadian resting phase, and half of the animals were trained in the last hour of the light phase, i.e., just before the start of their active phase. The second time point was chosen to avoid having to expose these animals to light when they normally would not perceive, which would have been the case if animals had been trained at the beginning in the dark phase. Both groups of mice were injected with a protein synthesis inhibitor (see below) or vehicle solution either directly ("T0"), 4 hours after training ("T4"), 8 hours after training ("T8"), or 11.5 hours after training ("T11.5"). In all cases, the CFC test trial occurred 24 hours after the initial training. For an overview of the experimental design, see Figure 1.



▲ **Figure 1. Experimental design.**

Animals are either trained at the beginning of the light phase ("light phase") or at the end of the light phase ("dark phase"). Animals are trained in the contextual fear conditioning task where they receive a shock of 0.75mA. After the shock animals are injected subcutaneously with vehicle or anisomycin, either directly after training, 4 hours, 8 hours, or 11.5 hours following training. Twenty-four hours after the fear conditioning training, animals are re-exposed to the same training context to measure freezing levels. ANI: Anisomycin. SC: subcutaneous.

Habituation and fear conditioning

Three consecutive days prior to the CFC task, animals were transported to the experimental room and handled by the experimenter for 2 minutes a day. On the last day of habituation, animals were weighed and received a tail mark with a black permanent marker for identification. Furthermore, the day before CFC training, mice also received a subcutaneous mock injection of 50µl 0.9% saline in order to habituate them to this procedure.

Animals were trained in the CFC task using a foreground conditioning protocol (Havekes et al., 2012; Havekes et al., 2016a). On the training day, mice were placed in the fear conditioning box (Ugo Basile). After 2.5 minutes, mice were subjected to a single 2-seconds foot shock of 0.75 mA. Thirty seconds following the shock, mice were returned to the housing chamber and received a subcutaneous injection of anisomycin or vehicle at a specific time point as shown in Figure 1. Twenty-four hours after training, animals were re-exposed to the conditioning box for 5 consecutive minutes, without the delivery of a shock. Before the training and test session of each new mouse, the fear conditioning box was cleaned with 70% ethanol. Fear conditioning was assessed by scoring freezing behavior, defined as a complete lack of movement except for respiratory behavior, which was determined using EthoVision XT software (Noldus Information Technology). This software yields reliable

measurements of freezing behavior (Pham et al., 2009).

Drug treatment

Animals were injected subcutaneously with anisomycin (150 mg/kg; A&E Scientific, Marcq, Belgium) to temporally inhibit protein synthesis, or with equivalent volume of vehicle solution (0.9% saline). Anisomycin was dissolved in 0.9% saline using 3.7% HCl, after which the pH was adjusted to 7.4 using 4% NaOH. At the concentration used, anisomycin inhibits cerebral protein synthesis in mice for 15-45 min (Davis & Squire, 1984). Injections were performed in the housing room by an experimenter different from the one who performed the behavioral task.

Statistics

Delta freezing levels were obtained by normalizing the animal's freezing behavior during the test to baseline (= pre-shock) freezing levels. Differences in normalized freezing behavior between the anisomycin-injected mice and control mice per phase and time-point were calculated with ANOVA. A one-way ANOVA was used to calculate differences between time-points for the vehicle-injected animals per phase. A one-way between subjects ANCOVA was used to test for the effect of sex. Additionally, to calculate the relative differences between anisomycin and vehicle injected animals, we first averaged the mice injected with vehicle per time point, per phase. Then, we subtracted all individual anisomycin data points by their corresponding vehicle average. A two-way ANOVA, using time point and phase as independents, and anisomycin normalized to vehicle freezing levels as dependent, was performed to examine whether the effect of anisomycin on freezing depended on time point of injection, (2) light or dark phase, and (3) whether there was an interaction effect of time point and phase.

Five animals showed very high freezing levels during the pre-shock training interval, i.e., more than 2 SD above the group mean, and were therefore excluded from the analysis (SPSS extreme value analysis). These five animals belonged to the groups: Light phase, T0, Anisomycin; Dark phase, T4, Anisomycin; Dark phase, T8, Vehicle; Dark phase, T8, Anisomycin; Dark phase T11.5, Anisomycin. Data are presented as mean \pm SEM, including a pirateplot showing the spread of the individual data points. Statistical analy-

ses were performed using SPSS 25.0 software (Armonk, NY, USA: IBM Corp). Data figures were produced in R (Boston, MA, USA), using the yarr package (Phillips, 2017). Differences were considered statistically significant when $p < 0.05$.

Results

We first examined at which time points protein synthesis inhibition impaired hippocampus-dependent memory consolidation when the animals were trained in the beginning of the light phase (Figure 1). Animals received subcutaneous vehicle or anisomycin injections after contextual fear conditioning and 24 hours after training, animals were re-exposed to the CFC chamber and freezing levels were measured. No differences were found in pre-shock freezing levels during the training at the beginning of the light phase (T0: Control $0.9 \pm 0.3\%$ ($n=8$), Anisomycin $0.7 \pm 0.3\%$ ($n=7$), ANOVA $F < 1$; T4: Control $0.7 \pm 0.2\%$ ($n=8$), Anisomycin $1.7 \pm 0.7\%$ ($n=8$), ANOVA $F = 1.7$; T8: Control $1.3 \pm 0.3\%$ ($n=8$), Anisomycin $1.2 \pm 0.4\%$ ($n=8$), ANOVA $F < 1$; T11.5: Control $1.6 \pm 0.4\%$ ($n=8$), Anisomycin $0.8 \pm 0.2\%$ ($n=8$), ANOVA $F = 2.9$; see also supplementary figure 1A).

Subsequently, we calculated the delta freezing levels by normalizing the animal's freezing behavior during the test to baseline (pre-shock) freezing levels. As indicated in Figure 2, anisomycin successfully inhibited CFC memory consolidation when injected directly, 4 hours and 8 hours after training (respectively, $F_{1,14} = 24.6$, $p < 0.001$; $F_{1,15} = 6.4$, $p < 0.05$; $F_{1,15} = 11.2$, $p < 0.01$; Figure 2A). However, anisomycin did not impair memory consolidation when injected 11.5 hours following training ($F_{1,15} = 0.1$, $p > 0.5$; Figure 2A). Importantly, there was no difference in freezing levels between the different time points for the vehicle-injected animals ($F_{3,31} = 0.9$, $p > 0.1$; Figure 2A). Hence, protein synthesis inhibition directly following training, or 4, and 8 hours following training effectively impaired memory consolidation in the light phase. In contrast, delivery of the protein synthesis inhibitor at 11.5 hours after training did not impact the consolidation of context-fear memories.

In the next set of studies, we examined whether protein synthesis inhibition impaired memory consolidation at the same time points following training when training was conducted just before the onset of the dark phase, i.e., the animals' active phase (Figure 1). Again, animals did not differ in pre-shock

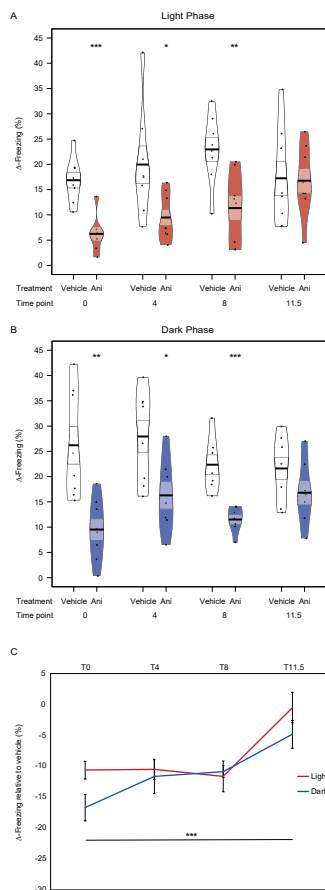
freezing levels during the training at the end of the light phase (T0: Control $2.4 \pm 0.6\%$ (n=8), Anisomycin $2.3 \pm 0.6\%$ (n=8), ANOVA $F < 1$; T4: Control $2.7 \pm 0.8\%$ (n=8), Anisomycin $4.6 \pm 0.7\%$ (n=7), ANOVA $F = 2.9$; T8: Control $0.8 \pm 0.2\%$ (n=7), Anisomycin $1.6 \pm 0.5\%$ (n=7), ANOVA $F = 1.5$; T11.5: Control $1.1 \pm 0.3\%$ (n=8), Anisomycin $0.8 \pm 0.2\%$ (n=7), ANOVA $F < 1$; see also supplementary figure 1B).

As can be seen in figure 2B, delivery of anisomycin directly, 4 hours and 8 hours after training during the dark phase inhibited CFC memory consolidation (respectively, $F_{1,15} = 14.8$, $p < 0.01$; $F_{1,14} = 7.4$, $p < 0.05$; $F_{1,13} = 23.9$, $p < 0.001$; Figure 2B). However, when injected 11.5 hours after training, anisomycin did not result in a memory deficit ($F_{1,15} = 1.0$, $p > 0.1$; Figure 2B). In addition, the vehicle-injected animals showed no difference in freezing levels between the different time points of injection ($F_{3,30} = 1.1$, $p > 0.1$; Figure 2B). Therefore, these data indicate that inhibition of protein synthesis directly after training as well as 4, and 8 hours following training attenuates memory consolidation, in the dark and light phase in a similar fashion.

In addition, we calculated the relative differences between anisomycin and vehicle injected animals by first averaging the mice injected with vehicle per time point, per phase. Then, we subtracted all individual anisomycin data points by their corresponding vehicle average and plotted this for both the light and dark phase (Figure 2C). In line with the previous findings, there is a significant effect of time point of injection, indicating that the effect of anisomycin on freezing levels depends on the time when administered ($F_{3,52} = 10.1$, $p < 0.001$; Figure 2C). In addition, there is no general effect of phase, suggesting that the delta freezing levels do not differ between the light and dark phases ($F_{1,52} = 3.2$, $p > 0.05$; Figure 2C). Furthermore, there is no interaction effect between phase and time point of injection ($F_{3,52} = 1.0$, $p < 0.1$; Figure 2C). Altogether, this additional analyses suggest that the effect of anisomycin on the delta freezing levels only depends on the time point of injection, irrespective of phase, which is in accordance with our conclusion based on the analyses above.

Finally, we assessed whether sex differences influenced freezing levels and response to protein synthesis inhibition. We first performed a one-way between subjects ANCOVA to examine the effect of protein inhibition on freez-

ing levels during the test phase (i.e., 24 hours after the training) controlling for the effect of sex. Analysis indicated that sex was not significantly related to freezing levels ($F_{1, 120} = 2.7, p > 0.1$; data separated for sexes not shown). Altogether, protein synthesis inhibition decreased freezing levels in both phases in a similar fashion independent of sex.



▲Figure 2. Protein synthesis inhibition attenuates the consolidation of context-fear memories at specific time points following training, irrespective of time of day.

Animals were trained in the contextual fear conditioning paradigm either at the beginning or the end of the light phase. Injections were given either directly (T0), 4 hours (T4), 8 hours (T8), or 11.5 hours (T11.5) after training. Animals were re-exposed to the same context 24 hours after training during which freezing levels were measured. Then, delta-freezing levels were calculated by normalizing freezing levels during the test to baseline (pre-shock). A) Delta-freezing levels after contextual fear training at the beginning of the light phase and drug treatment during the light phase. T0: vehicle n=8, Anisomycin n=7 ($p < 0.001$); T4: vehicle n=8, Anisomycin n=8 ($p = 0.024$); T8: vehicle n=8, Anisomycin n=8 ($p = 0.005$); T11.5: vehicle n=8, Anisomycin n=8 ($p = 0.912$). B) Delta-freezing levels after contextual fear training at the end of the light phase and drug treatment during the dark phase. T0: vehicle n=8, Anisomycin n=8 ($p = 0.002$); T4: vehicle n=8, Anisomycin n=7 ($p = 0.017$); T8: vehicle n=7, Anisomycin n=7 ($p < 0.001$); T11.5: vehicle n=8, Anisomycin n=7 ($p = 0.168$). C) Delta-freezing levels in anisomycin treated animals relative to vehicle treated animals. T0: Light phase n=7, Dark phase n=8; T4: Light phase n=8, Dark phase n=7; T8: Light phase n=8, Dark phase n=7; T11.5: Light phase n=8, Dark phase n=7.

Panel A & B: data are expressed as the mean, the area (band) around the mean indicates SEM, the smoothed density curve (bean) shows the full data distribution, dots indicate individual data points. Freezing behavior was assessed during 300s. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as calculated by ANOVA. Panel C: All data are expressed as the mean \pm SEM. Freezing behavior was assessed during 300s. Time point *** $p < 0.001$, Phase $p = 0.081$, interaction effect $p = 0.386$, as calculated by a two-way ANOVA.

Discussion

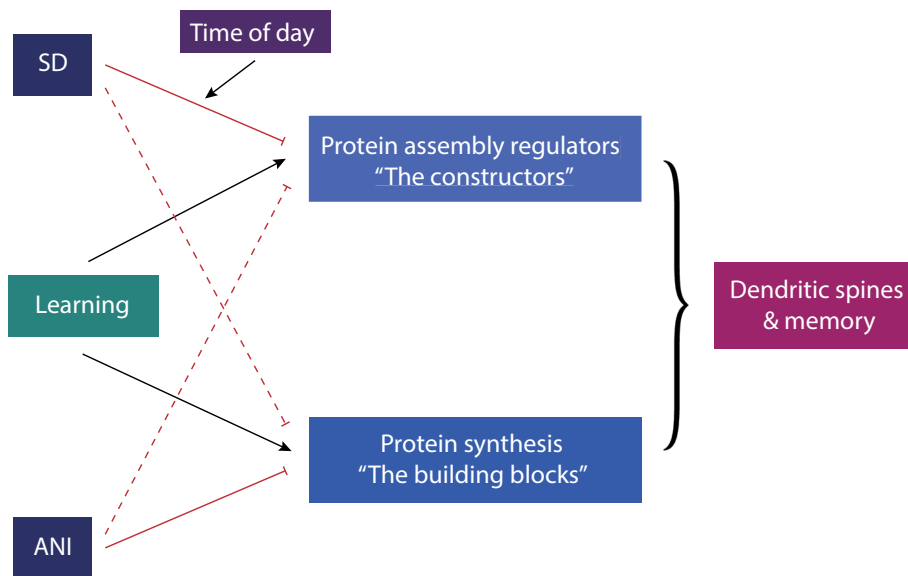
This study aimed to gain insight into the role of protein synthesis in the consolidation of hippocampus-dependent memories across different phases of the 24-hour cycle. For this purpose, we trained mice in the CFC task either at the beginning or end of the light phase and inhibited protein synthesis at different time points following training. We found that protein synthesis inhibition impaired CFC memory consolidation independent of the timing of training, directly, 4 hours and 8 hours following training. These data underscore the importance of *de novo* protein synthesis for the consolidation of contextual fear memories and suggest that the temporal dynamics of protein synthesis-dependent consolidation of contextual-fear memories is similar across the light and dark phase.

Interestingly, we found that blocking protein synthesis 8 hours after CFC training at the beginning of the light phase impaired memory consolidation. This finding is somewhat surprising as previous work revealed that CFC memory is selectively affected by inhibition of protein synthesis either directly or 4 hours, but not 8 hours after training (Bourtchouladze et al., 1998). Regarding this discrepancy, it should be noted that Bourtchouladze and colleagues used a background conditioning rather than foreground conditioning protocol. During background conditioning, the US is paired with a tone, which acts as a second CS in addition to the training context. Thus, subtle differences in the CFC training protocol (in this case the presence or lack of a tone during the shock) may alter the temporal dynamics of protein synthesis-dependent memory consolidation. Indeed, the use of a more robust background conditioning training protocol (i.e., with multiple shocks) results in the consolidation of context-fear memories that is disrupted by anisomycin treatment immediately following training, but not at the four-hour time point (Bourtchouladze et al., 1998). However, in line with our observations, Trifilieff et al. showed that CFC training leads to an activation of the ERK/CREB pathway in the CA1 region of the hippocampus approximately nine hours following training (Trifilieff et al., 2006). This activation may contribute to the *de novo* synthesis of proteins that are crucial for the consolidation of contextual fear memories. In future studies, it would be interesting to examine how subtle alterations in CFC training protocols affect the temporal dynamics of protein synthesis-dependent memory consolidation and activation patterns of the related signaling pathways.

As mentioned previously, the activity of signaling pathways involving MAPK, cAMP, and PKA are critical for learning and memory and vary across the circadian cycle (Eckel-Mahan et al., 2008). Whereas some studies examined baseline circadian rhythms of signaling pathways, other studies focused on learning-induced plasticity. Background conditioning, for example, is associated with a single phase of activation of the ERK/CREB pathway in the CA1 region of the hippocampus directly after training. Unpairing of the tone and the US – i.e., when the tone is given pseudorandomly, not together with the shocks – leads to a paradigm that is more comparable to foreground conditioning and the protocol that was used in this study. This results in two distinct waves of ERK/CREB activation in the CA1 region: one directly after training and the second approximately nine hours after training (Trifilieff et al., 2006). Inhibition of ERK/CREB during any of these phases was sufficient to impair memory formation after unpaired fear conditioning. As stated in the previous paragraph, the timing of these two waves of ERK/CREB activation appears to be comparable to the time points directly and eight hours after contextual fear training in this study, at which the consolidation of contextual fear memories depends on *de novo* synthesis of proteins. Thus, ERK/CREB signaling may contribute to the *de novo* synthesis of proteins that are crucial for the consolidation of contextual fear memories directly or 8-9 hours after training. Protein synthesis at the 4-hour time point may be orchestrated by other ERK-independent mechanisms such as the PKA-CREB pathway. Indeed, intrahippocampal injection with Rp-cAMPs (a PKA inhibitor) at 4 hours following fear conditioning impairs the consolidation of context-fear memories (Bourtchouladze et al., 1998). The notion of two waves of protein synthesis is supported by another study which used a motor learning paradigm (Peng & Li, 2009).

It is important to note that it is challenging to separate the circadian, and/or time-of-day effects on memory consolidation from changes in the sleep/wake cycle (Snider et al., 2018). Sleep has a strong influence on memory processes, and hippocampus-dependent memories are vulnerable to sleep loss (Graves et al., 2003; Vecsey et al., 2009; Havekes et al., 2014; Havekes et al., 2016b; Raven et al., 2017). For example, 5-6 hours of sleep deprivation (SD) directly after CFC training impairs memory consolidation in both mice and rats (Graves et al., 2003; Vecsey et al., 2009; Hagewoud et al., 2010; Kreutzmann et al., 2015). Importantly, these studies were conducted in the

light phase, which is the main resting phase of sleep phase of laboratory rats and mice. In contrast, six hours of sleep deprivation directly following training at the beginning of the dark phase, the active phase in which rodents sleep far less, did not impair memory consolidation (Hagewoud et al., 2010). Only when animals were deprived of sleep for twelve hours, spanning the entire dark period, it hampered the formation of long-term CFC memories (Hagewoud et al., 2010). While these findings suggested that the temporal regulation of the molecular processes underlying hippocampus-dependent memory consolidation may differ across the active and the inactive phase, dependent on the amount of sleep in these phases, they raise the question how it is possible that protein synthesis inhibition affects memory consolidation independent of time of day. Does it imply that the negative consequences of sleep loss are at least in part protein synthesis independent? For a memory to come to exist, an initial memory trace needs to be made from de novo proteins, eventually forming actin filaments creating new spines, hence forming new synaptic connections. Therefore, inhibition of protein synthesis directly hampers memory consolidation as new memories cannot be formed. This process involves, and is sensitive to, many molecular constructors modulating the formation of new synapses. One example of such a molecular constructor is cofilin. Cofilin is an actin destabilizing protein and when activated, disassembles actin filaments causing loss of dendritic spines (Bamburg & Wiggan, 2002; Bernstein & Bamburg, 2010). A short period of SD in the light phase increases cofilin activation eventually causing spine loss (Havekes et al., 2016b). Furthermore, SD reduces mTORC1 activity signaling in the hippocampus, affecting translation, thereby also indirectly reducing protein synthesis, potentially resulting in memory impairments (Tudor et al., 2016). Together, SD impairs important molecular mediators of synaptic plasticity, and thereby indirectly affecting memory storage. This could explain why SD only impairs memory in the light phase, when sleep pressure is high, targeting the constructors instead of the fundamental building blocks of memories itself (Figure 3).



▲ **Figure 3. A hypothetical model describing how sleep deprivation and protein synthesis inhibition impact memory consolidation.**

Long-term memory requires synthesis of *de novo* proteins, which are the building blocks of dendritic spines, eventually forming new synaptic connections. Inhibition of *de novo* protein synthesis during sensitive periods therefore directly impairs long-term memory. On the other hand, the building blocks need to be assembled by protein assembly regulators or “builders” such as actin (de)stabilizers. When the balance between regulators is disturbed during sensitive periods, for example as a result of sleep deprivation, memory can be impaired. ANI, Anisomycin; SD, Sleep deprivation.

Given that sleep loss has a strong effect on learning and memory (Havekes & Abel, 2017; Raven et al., 2017), one might wonder if the effects of anisomycin on memory formation are not only directly caused by inhibition of protein synthesis, but perhaps in part indirectly by affecting sleep. Although in our study we did not measure EEG or motion during the course of the experiment, literature shows that anisomycin can have subtle effects on sleep. For example, whereas non-rapid eye movement (NREM) sleep is more often reported to be unaffected, injections of anisomycin might decrease rapid eye movement (REM) sleep (Rojas-Ramirez et al., 1977; Drucker-Colin et al., 1979; Gutwein et al., 1980). Yet, it is uncertain whether these effects on sleep are strong enough to explain the memory deficits in our study. Moreover, such an indirect effect of anisomycin on memory through changes in sleep is not supported by the results of the injections at the end of the dark phase, just before the start of the main circadian sleep phase. If anisomycin would have major effects on sleep that could impair memory formation, one would

expect this to have occurred with injections at that time point as well, which was not the case.

Although, as mentioned previously, the indirect effects of anisomycin on memory are largely unknown, it remains one of the most widely used tools to manipulate protein synthesis in order to investigate memory processes (Davis & Squire, 1984; Rudy et al., 2006; Gold, 2008). Using anisomycin, researchers found that protein synthesis is necessary for long-term memories to persist. Several studies have been performed applying anisomycin locally and therefore increasing spatial resolution, combined with its already advantageous temporal resolution. Recently, Ryan et al. (2015) showed that anisomycin impaired memory, but that this amnesia could be recovered, thereby implying that protein synthesis is important for structural strengthening of the synapse, which is necessary during memory retrieval (Ryan et al., 2015). One disadvantage of anisomycin is that it is also capable of disrupting basic neurobiological functions, for example by causing apoptosis (Iordanov et al., 1997), which can affect the neurons' well-being and thereby contribute to memory impairments. For example, a few studies showed that anisomycin hampered basic membrane properties of hippocampal neurons (Sharma et al., 2012; Scavuzzo et al., 2019). Therefore, the memory impairments seen after injections of anisomycin could also be influenced by anisomycin-induced alterations in neural activity. However, in our study, we observed no effects of anisomycin at the latest time point, which indicates that if anisomycin reduced basic cell properties, these effects are almost negligible in our paradigm of the contextual fear conditioning task. Nevertheless, the use of other more specific inhibitors, such as rapamycin, which specifically targets mTORC1 is advised, to largely rule out any effects on basic cell functioning.

Knowledge on the dynamics of memory consolidation across the day and night is of great importance for all studies that aim to unravel the molecular mechanisms underlying memory formation. Future research should examine whether other molecular mechanisms supporting memory and known to be susceptible to SD are differently affected across the day and night. These may include transcription, translation, RNA-binding and ubiquitination, as revealed by a genomic analysis (Vecsey et al., 2012). Furthermore, future studies can utilize novel and more sophisticated techniques, such as optogenetics to tag neurons in an activity-dependent way to see how and when certain

memories are formed and preserved in time (Ryan et al., 2015; Tonegawa et al., 2015). These technological advances may lead to a better understanding of the molecular underpinnings of hippocampus-dependent memory consolidation across day and night.

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Abbreviations

CFC, Contextual fear conditioning	MAPK, mitogen-activated protein kinase
CREB, cAMP response element-binding protein	NREM, non-rapid eye movement
CS, conditioned stimulus	PKA, protein kinase A
mTORC1, mammalian target of rapamycin complex 1	REM, rapid eye movement
	SD, sleep deprivation
	US, unconditioned stimulus

Conflict of Interest Statement

None

Author Contributions

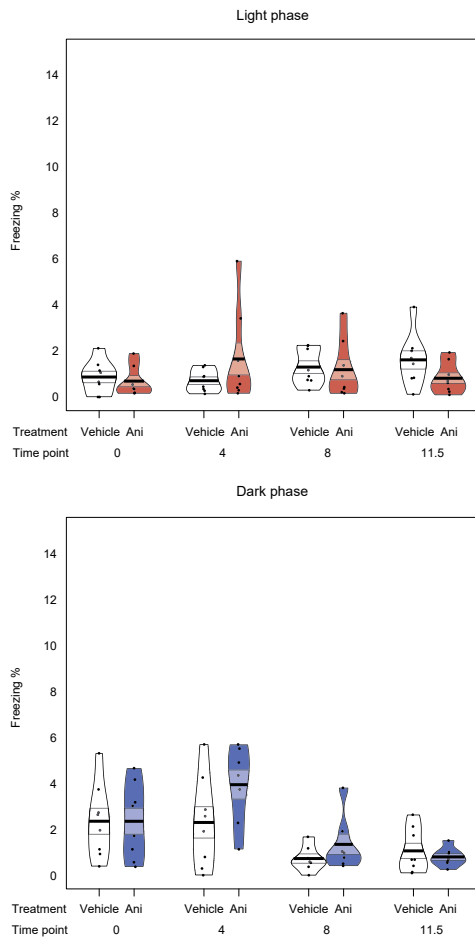
Design of the experiment: FR, EAvdZ, PM, RH

Execution of the experiments: FR, YGB, LVvR, ELM

Writing of the manuscript: FR, YGB, LVvR, PM, RH

Data Accessibility Statement

All data, including the fear conditioning videos, output and SPSS data files will be stored according to the research data management plan (RDMP) at the Groningen Institute for Evolutionary Life Sciences (GELIFES) at the University of Groningen, The Netherlands. Data will be made available upon request.



▲ Supplementary Figure 1. Pre-shock freezing levels do not differ between groups.

A) Freezing responses during contextual fear conditioning training before receiving the shock, at the beginning of the light phase. T0: vehicle $n=8$, Anisomycin $n=7$ ($p = 0.628$); T4: vehicle $n=8$, Anisomycin $n=8$ ($p = 0.218$); T8: vehicle $n=8$, Anisomycin $n=8$ ($p = 0.832$); T11.5: vehicle $n=8$, Anisomycin $n=8$ ($p = 0.110$). B) Freezing responses during contextual fear training before receiving the shock, at the end of the light phase. T0: vehicle $n=8$, Anisomycin $n=8$ ($p = 1.0$); T4: vehicle $n=8$, Anisomycin $n=7$ ($p = 0.111$); T8: vehicle $n=7$, Anisomycin $n=7$ ($p = 0.237$); T11.5: vehicle $n=8$, Anisomycin $n=7$ ($p = 0.516$). Freezing behavior was assessed during 2.5 minutes. Data are expressed as the mean, the area (band) around the mean indicates SEM, the smoothed density curve (bean) shows the full data distribution, dots indicate individual data points.

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